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ABSTRACT

Bovine Corneal Opacity and Permeability Test Validation as an Alternative to the Draize Eye Irritation Assay.

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The Bovine Corneal Opacity and Permeability Assay (BCOP) has been proposed for use as an alternative to the Draize eye irritation assay. In this study we evaluated the *in vitro* scores for the BCOP assay in relation to those obtained using abbreviated 3 rabbit Draize eye irritation assays and the chorioallantoic membrane vascular assay (CAMVA). The products and chemicals used in this evaluation were chosen based on their Draize irritation potential and included dilutions of SDA-40 alcohols, alcohol containing products, cosmetic products and shampoos. Draize Mean Total Scores ranged from 0 to 48.33. RC_{50} values from the CAMVA ranged from 1.0 to >100 and *in vitro* scores from the BCOP ranged from < 0 to 60.68. The data suggest that both the BCOP and CAMVA assays can be used as screens for ocular irritation potential. However, the BCOP may be more accurate at low irritancy levels.

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INTRODUCTION

Both the Chorioallantoic Membrane Vascular Assay (CAMVA) and the Bovine Corneal Opacity and Permeability (BCOP) tests have been developed as alternatives to the use of laboratory animals for ocular irritation evaluations [1-7]. In the CAMVA the vascular responses of the chorioallantoic membrane (CAM) of fertile DeKalb eggs are evaluated 30 minutes after treatment. The RC_{50}, that concentration which produces vascular responses in 50% of the treated eggs, is determined by probit analysis and serves as an index of ocular irritancy. The *in vitro* score is calculated from the responses of excised bovine corneas and consists of two parts, the opacity measurement and the permeability measurement.

MB Research Laboratories has been validating the two alternative assays. Previous evaluations [3,4] dealt strictly with the CAMVA assay. The objective of this study was to compare the BCOP test with both the CAMVA and the Draize ocular irritation assay. Four classes of materials were tested, i.e., cosmetic products, shampoos, glycols and alcohol or alcohol-containing products.

The RC_{50} values and the *in vitro* scores were compared to the mean day 1 Draize scores from 3 rabbit eye irritation evaluations. Previous evaluations of CAMVA studies resulted in RC_{50} ranges which corresponded to the Draize classification of irritant, non-irritant and indeterminate. Although Gautheron, et al., 1992[5] proposed irritancy levels based on BCOP *in vitro* scores, it was our intention to further classify the irritancy potential of the four classes of materials tested.

TEST MATERIALS

A total of 17 cosmetic products were tested including eye shadows, mascara, makeups, sun block, makeup remover, facial scrub and blushing gel.

Ten alcohol dilutions or alcohol-containing materials were tested including SDA-40-2, after shave products, pre-shave products and an astringent.

Three glycol products were evaluated: butylene, propylene and hexylene glycol.

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DRAIZE METHOD

Three healthy New Zealand white rabbits, free from evidence of ocular irritation and corneal abnormalities, were dosed with each product or product dilution. A dose of 0.1 ml was placed by syringe into the conjunctival sac of one eye of each animal after gently pulling the lower eyelid away from the eye. After instillation, the lids were held together for approximately 1 second to insure adequate distribution of the test article.

Each treated eye was examined for irritation of the cornea, iris and conjunctiva on days 1, 2 and 3 following dosing. Ocular reactions were graded according to the numerical Draize technique (Table 1)[8]. Additional signs were described.

The primary eye irritation score for each rabbit was calculated from the weighted Draize scale (Table 1) and the Mean Total Score (MTS) for each day was determined by averaging the individual primary eye irritation scores.

CAMVA METHOD

The 14 day incubation CAMVA method was selected rather than the 10 day because we have had more consistent results with the former[9].

Fertile DeKalb XL strain eggs were selected for each evaluation from a larger group received from Moyer's Chicks, Quakertown, PA. The eggs were kept in incubators at 99 ± 2° Fahrenheit and 50 - 60% relative humidity. During the incubation period, the position of the egg tray within the incubator was changed daily to insure even atmospheric exposure.

On Day 4 of the incubation period, the eggs were removed from the incubator and candled to determine the presence and location of the embryos. After determining the presence and marking the location of the embryo, a small hole was drilled into the narrow end of each egg using a dentist's drill with a diamond wheel bit. Approximately 2.5 ml of albumin was removed using a needle and syringe in order to lower the CAM sufficiently to prevent damage and allow an open area for treatment and examination. The hole was sealed with collodion adhesive. A rectangular window was cut using a dental drill and then removed with forceps. The opening was covered with transparent tape. The eggs were returned to the incubator for the remainder of the 14 day period. On Day 14, the eggs were removed from the incubator, the tape peeled back and the CAM examined for any abnormalities. Any egg with improperly developed membranes, undeveloped membranes or any other abnormality was discarded.

Following the pre-dose examination, a Teflon ring was gently placed on the CAM and 40 µl of the product or dilution was pipetted into the ring. The window was then resealed, the egg numbered and returned to the incubator. After 30 ± 5 minutes, the eggs were removed from the incubator and the CAM exposed by removing the tape and portions of the surrounding shell. The condition of the CAM within the Teflon ring was examined and recorded. Vascular hemorrhage, capillary injection and/or the presence of ghost vessels was considered a positive response. If any abnormalities were noted outside the ring, the egg was not included in the calculations. Ten eggs were used for each dilution. At least four dilutions of each product or SDA-40 were evaluated.

The percentage of CAM's responding positively to each dilution were plotted on 3 cycle log-probit paper and an RC_{50} (the calculated concentration theoretically producing a positive reaction in 50% of the treated eggs) with 95% Confidence Limits was calculated using the method of Litchfield and Wilcoxon[10]

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BCOP METHOD

The bovine eyes were received from a local supplier and transported to MB Research Laboratories in Hanks Balanced Salt Solution in a refrigerated container. The eyes were examined within one hour after receipt and any cornea exhibiting evidence of vascularization, pigmentation, opacity or scratches was discarded.

Corneas which were free of defects were dissected from the surrounding tissues. A 2-3 mm rim of sclera was left attached to each cornea. The dissected corneas were mounted in specially designed holders segmented into anterior and posterior chambers which were filled separately. Each cornea was mounted allowing the epithelium of the cornea to project into the anterior chamber. The posterior chamber was filled with Minimal Essential Media supplemented with 1% fetal bovine serum (MEM). The anterior chamber was then filled with MEM. Each cornea was visually inspected again to insure that there were no defects. The entire holder with the cornea was submerged in a 32°C water bath and allowed to equilibrate for at least one hour, but not longer than 2 hours.

Following equilibration, the holders containing the corneas were removed from the water baths. The MEM was removed from both chambers and the chambers refilled with fresh MEM. At this time, five corneas were selected for dosing with the test material and two were selected as controls.

Measurements of opacity through the cornea were made using an OP-KIT™ opacitometer produced by Electro-Design Corporation of Rion, France. At each interval, each treated cornea was scored and compared to the two control corneas. A pre-exposure determination of opacity was made for each control by measuring each against the blanks supplied with the opacitometer. A pre-exposure determination of opacity was made for each of the 5 test corneas by measuring against each control cornea (a total of 10 determinations).

Following the pretest observations, the MEM was removed from the anterior chamber and a volume of 0.75 ml of the undiluted test material was applied to the epithelium of each of the five treated corneas. The holders and corneas were then placed in the 32°C water bath in a horizontal position to insure contact of the test material with the cornea. After 10 ± 1 minute, the test substance (or MEM in the controls) was removed from the epithelium of the cornea and the anterior chamber by washing with MEM. All holders were then refilled with fresh MEM, returned to the water bath and incubated at 32°C for an additional two hours. At the end of the two hour period, the MEM was changed again and a measurement of opacity taken comparing each of the five treated corneas to the two control corneas. Immediately following the two hour opacity measurement, the MEM was changed in the posterior chamber of both the control and test corneas. The MEM was removed from the anterior chamber and replaced with 1.0 ml of 0.4% sodium fluorescein solution in both the treated and control corneas. Fresh holders and corneas were then returned to the 32°C water bath in a horizontal position to insure contact of the fluorescein with the cornea.

After 90 minutes, the fluid from the posterior chamber was removed and the amount of dye which passed through the cornea was measured as the optical density at 450 nm using a Spectronic 20 Spectrophotometer.

When the test material was a solid, it was dissolved in MEM at a 20% dilution and allowed to remain in contact with the cornea for 4 hours rather than 10 minutes. The opacity measurement was taken after the 4 hour exposure. When the test material was known to contain alcohol, an additional opacity measurement was taken when the test material was removed following the 10 minute exposure.

The corrected mean opacity score was calculated using the control and treated cornea opacity values as determined from the OP-KIT. The corrected mean optical density score was calculated using the control and treated optical density values from the fluorescein permeability analysis. The *in vitro* score was calculated as:

Corrected Mean Opacity Score + 15 (Corrected Mean Optical Density Score).

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RESULTS

The CAMVA RC_{50}'s, BCOP *in vitro* scores and corresponding day 1 Draize Mean Total Scores are presented in Table 2. The results of the Draize ocular testing were also classified for levels of irritancy according to a modification of the original Draize interpretation using only 3 animals as follows:

Non-Irritant	0 rabbit with positive scores
Indeterminate	1 rabbit with positive scores
Irritant	2 - 3 rabbits with positive scores

The CAMVA RC_{50}'s ranged from 11 to >100% for cosmetic products, 11.0 to >100% for alcohol and alcohol containing materials, 0.19 to 14% for shampoos and 7.6 to 30% for glycols.

The BCOP *in vitro* scores ranged from -0.52 to 41.35% for cosmetic products, -4.25 to 45.38% for alcohol and alcohol containing materials, -3.92 to 60.6% for shampoos and 1.02 to 21.45% for glycols.

Day 1 Draize Mean Total Scores ranged from 0 to 30.0 in cosmetic products, 0 to 48.33 for alcohol and alcohol containing materials, 0 to 18.67 for shampoos and 0 to 16.67 for glycols.

The Draize values may have overestimated the true ocular responses for D-51 and D-52 eye shadows and D-20 antiperspirant since it was noted in the day 1 observation that the material remained in the conjunctiva. The increased exposure may have resulted in elevated ocular responses. Additionally, the 8.67 day 1 Draize score for D-10 blushing gel may have been the result of staining of the conjunctiva rather than an erythematous response.

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DISCUSSION

We conducted these evaluations to classify the irritancy potential of classes of test materials according to their *in vitro* scores using the BCOP assay. Information supplied with the opacitometer suggested the following classification scheme:

In-Vitro Score	Classification
0 to 25	Mild Irritant
25.1 to 55	Moderate Irritant
55.1 and greater	Severe Irritant

Of particular interest was delineating the mild irritant category, i.e. determining the *in vitro* scores which correspond to the limits of the irritant/non-irritant category of the Draize evaluations.

For cosmetic products the *in vitro* score which approximates the boundary between irritant and non-irritant appears to be in the 4 to 5 unit range. Although it appears from Table 2 that a number of materials would be false negatives, assuming a boundary of 4 or 5, it is likely that for two test articles, D-20 and D-52, the Draize values may be overestimations of the true irritancy potential. In both instances, the powder materials remained in the conjunctiva for 24 hours. The prolonged exposure may have resulted in increased Draize Mean Total Scores. The blushing gel, D-10, may also have been a false positive since reddening of the conjunctiva was the only abnormal ocular effect noted in the rabbits and may have been staining rather than an erythematous reaction. The sun block, D-4, and the facial scrub, D-5, were the only other false negatives and will be re-examined in the future.

CAMVA RC_{50}'s for the cosmetic products were all above the irritant/non-irritant boundary of 1 to 3% found for surfactants[4] and indicated that it may be necessary to validate this study in more detail when using different chemical entities.

In the alcohol group of test materials, there were fewer Draize responses in the borderline range. The irritant/non-irritant interface for the BCOP assay appears to be in the range of approximately 5 or 6 units. Previous evaluations[4] indicated that the RC_{50} values of less than 30% corresponded to eye irritants and RC_{50} values greater than 40% corresponded to little or no irritation.

Among the neat shampoos, only the baby shampoo was classified as non-irritating. In addition, two aqueous shampoo dilutions were in the non-irritating category and one dilution was indeterminate. These data indicate that the irritant/non-irritant boundary for shampoos in the BCOP assay is between 2 and 10, but probably closer to 2. As previously published, the CAMVA RC_{50} limits were similar to those of surfactants[3], where the irritant/non-irritant boundary was approximately 1.0.

The results observed for cosmetic products indicated the BCOP may be a better test for use with products which tend to produce low levels of irritation.

SCALE FOR SCORING OCULAR LESIONS[1]

(1) CORNEA:

(A)	Opacity: Degree of density (area most dense taken for reading):		
	No ulceration or opacity		0
	Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible		1[2]
	Easily discernible translucent area, details of iris slightly obscured		2[2]
	Opalescent areas, no details or iris visible, size of pupil barely discernible		3[2]
	Opaque cornea, iris not discernible through the opacity		4[2]
(B)	Area of cornea Involved:		
	One quarter (or less) but not zero		1
	Greater than one-quarter, but less than one-half		2
	Greater than one-half, but less than three-quarters		3
	Greater than three quarters up to whole area		4
	SCORE EQUALS A x B x 5	**Maximum Total**	**80**

(2) IRIS:

(A)	Values:		
	Normal		0
	Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)		1[2]
	No reaction to light, hemorrhage, gross destruction (any or all of these)		2[2]
	SCORE EQUALS A x 5	**Maximum Total**	**10**

(3) CONJUNCTIVAE:

(A)	REDNESS (refers to palpebral and bulbar conjunctivae excluding cornea & iris):		
	Blood vessels normal		0
	Some blood vessels definitely hyperemic (injected)		1
	More diffuse, deeper crimson red, individual vessels not easily discernible		2[2]
	Diffuse beefy red		3[2]
(B)	CHEMOSIS		
	No swelling		0
	Any swelling above normal (includes nictitating membranes)		1
	Obvious swelling with partial eversion of lids		2[2]
	Swelling with lids about half closed		3[2]
	Swelling with lids more than half closed		4[2]
(C)	DISCHARGE		
	No Discharge		0
	Any amount different from normal (does not include small amounts observed in inner canthus of normal animals		1
	Discharge with moistening of the lids and hairs just adjacent to lids		2
	Discharge with moistening of the lids and hairs and considerable area around the eye		3
	SCORE EQUALS (A+B+C)x2	**Maximum Total**	**20**

The maximum total score is the sum of all scores obtained for the cornea, iris and conjunctivae.

[1] **Draize, J. H. et al. J. Pharm. Exp. Ther. 82:377-390, 1944.**

[2] **Indicates a positive response**

COSMETIC PRODUCTS

TEST MATERIAL	PRODUCT TYPE	DAY 1 DRAIZE MTS	DRAIZE CLASSIFICATION	RC_{50} (%)	BCOP *IN VITRO* SCORE
D-8	Eye Makeup Remover	0	negative	100	-0.52
D-6	Mascara	0	negative	>100	0.83
D-53	Nailcare Powder	0	negative	>100	-0.23
D-54	SPF-6 Suntan Cream	0	negative	87	2.11
D-55	SPF-6 Suntan Cream	0	negative	>100	2.23
D-50	Eye Shadow	0.67	negative	>100	1.07
D-51	Eye Shadow	0.67[1]	negative	>100	2.92
D-13	Blemish Control Makeup	0.67	negative	30	3.65
PRODUCT 21	Powder Makeup	2.00	indeterminate	>100	4.08
PRODUCT 22	Powder Makeup	2.33	positive	>100	4.49
D-20	Antiperspirant	4.67[1]	indeterminate	23	0.55
D-52	Eye Shadow	6.00[1]	positive	>100	0.81
D-4	Sun Block	6.00	indeterminate	>100	0.59
D-2	Cream Makeup	6.00	positive	103	5.72
D-5	Facial Makeup	6.67	positive	>100	1.10
D-10	Blushing Gel	8.67[2]	positive	11	0.55
D-56	SPF-15 Sun Screen Gel	30.00	positive	18	41.35

[1]Test material remaining in rabbit conjunctiva at 24 hours post dose.
[2]Red coloration of the conjunctiva may have been staining and not an irritant effect.

ALCOHOL and ALCOHOL CONTAINING MATERIALS

TEST MATERIAL	PRODUCT TYPE	DAY 1 DRAIZE MTS	DRAIZE CLASSIFICATION	RC_{50} (%)	BCOP *IN VITRO* SCORE
PRODUCT 6	After Shave Skin Conditioner	0	negative	>100	-4.25
PRODUCT 2	After Shave Conditioner	0	negative	56	2.23
30% SDA-40-2	Aqueous Dilution	0.67	negative	50	6.80
PRODUCT 3	After Shave	9.67	positive	20	24.18
50% SDA-40-2	Aqueous Dilution	11.33	positive	36	15.35
PRODUCT 5	After Shave	15.67	positive	22	45.38
70% SDA-40-2	Aqueous Dilution	16.67	positive	22	22.65
PRODUCT 8	Astringent	19.33	positive	16	44.28
PRODUCT 7	Preshave	23.33	positive	11	26.10
100% SDA-40-2	Full strength	48.33	positive	15	35.30

GLYCOLS

TEST MATERIAL	PRODUCT TYPE	DAY 1 DRAIZE MTS	DRAIZE CLASSIFICATION	RC_{50} (%)	BCOP *IN VITRO* SCORE
Butylene Glycol	Not applicable	0	negative	12.0	1.02
Propylene Glycol	Not applicable	2.0	negative	30.0	1.65
Hexylene Glycol	Not applicable	16.67	positive	7.6	21.45

SHAMPOOS

TEST MATERIAL	PRODUCT TYPE	DAY 1 DRAIZE MTS	DRAIZE CLASSIFICATION	RC_{50} (%)	BCOP *IN VITRO* SCORE
S-4 (10%)	Shampoo-Aqueous Dilution	0	negative	5.4	-3.92
S-1 (10%)	Shampoo-Aqueous Dilution	0.33	negative	14.0	1.35
BABY SHAMPOO	Undiluted	1.00	negative	3.6	2.10
S-5 (10%)	Shampoo-Aqueous Dilution	3.33	indeterminate	5.0	2.98
S-2	Shampoo-Undiluted	11.67	positive	0.32	10.60
S-1	Shampoo-Undiluted	12.00	positive	0.87	11.00
S-6	Shampoo-Undiluted	12.00	positive	0.99	21.83
S-3	Shampoo-Undiluted	12.33	positive	1.1	60.68
S-4	Shampoo-Undiluted	15.00	positive	1.0	13.80
S-5	Shampoo-Undiluted	18.67	positive	0.19	7.43

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